

Using microsatellite DNA to genetically identify a potential hybrid  
population of endangered massasauga rattlesnakes (*Sistrurus catenatus*)  
in north central Missouri

A Senior Honors Thesis

Presented in Partial Fulfillment of the Requirements for graduation  
*with research distinction* in Biological Sciences in the undergraduate  
colleges of The Ohio State University

by

Michael Joseph Murphy

The Ohio State University  
June 2009

Project Advisor: Professor H. Lisle Gibbs, Department of Evolution  
Ecology and Organismal Biology

## **Abstract:**

DNA-based genetic techniques can be used to identify individuals in wild populations whose ancestry is uncertain. The massasauga rattlesnake is a small rattlesnake found from central New York to southeastern Arizona. It is currently described as consisting of three subspecies: the eastern massasauga (*Sistrurus catenatus catenatus*), the western massasauga (*Sistrurus c. tergeminus*) and the desert massasauga (*Sistrurus c. edwardsii*). Due to morphological similarities between eastern and western massasaugas in north central Missouri (where the range of *S. c. catenatus* meets that of *S. c. tergeminus*), there is debate over the species identity of individuals in this population and the possibility that it is a hybrid population has been suggested. Here, I use microsatellite DNA from western and eastern massasaugas to determine the species identity of a central Missouri population. To do this, I used a combination of clustering analysis and distance-based phylogenetic analysis to determine the relationships among the western, eastern, and central Missouri populations. Cluster-based analysis using the program Structure showed that the central Missouri population clustered strongly with the western (*tergeminus*) populations. Distance-based phylogenetic analysis of populations also grouped the Missouri population with western populations and analysis of all individuals grouped the central Missouri individuals together with the western individuals. These results show that the western massasauga and central Missouri population are genetically similar, and both are highly differentiated from eastern massasauga populations. Therefore this data strongly suggests that the central Missouri population is *Sistrurus c. tergeminus*. This has the implications that one of the criteria for special protection status of this population has been removed and that the range of *S. c. catenatus* does not extend west of the Mississippi River which supports the possibility that the Mississippi River acts as a phylogeographical barrier for massasauga rattlesnakes.

## **Introduction:**

Natural hybridization is an enduring subject that evolutionary biologists have discussed extensively (Barton 2001, Mayr 1963, Arnold 1997). Evolutionary biologists focused much of their early hybridization research towards plants. Many argued that hybridization in plants was relatively widespread and that hybridization could support speciation (Grant 1971, Stebbins 1959, Anderson 1948, 1949, Rieseberg 1997, and Arnold 1996). The role of hybridization in the evolution of animal speciation traditionally has been disregarded as mostly insignificant except

in that it creates barriers to cross-species breeding (Dobzhansky 1937, Mayr 1963). More recently, research in animal hybridization has begun to gain momentum. Mallet (2005) suggested that evidence of hybridization exists in ten percent of known animal species. Evidence for animal hybridization has been cited in a variety of animal taxa from corals (Bette *et al.* 2006), to insects (Noor 2000, Carson *et al.* 1975, Post 1984, Della *et al.* 1997), fish (Crespin *et al.* 1999, Nielsen *et al.* 2003, Nolte *et al.* 2006), birds (Mank *et al.* 2004, Rohwer 2001, Saetre 2001) and mammals (Lehman *et al.* 1991, Lancaster *et al.* 2006, Schwartz *et al.* 2004, Trigo *et al.* 2008). Though there appears to be less documentation of hybridization in reptiles, some examples have been described (Seminoff *et al.* 2003, Cook 1955, Campbell *et al.* 1989).

Most of the claims of hybridization in reptiles (except Seminoff *et al.* 2003) are based on morphological similarities between the hybrid and both parental species. Mallet (2005) explains that describing a hybrid population based on morphological traits is difficult due to the possibility of polymorphisms in colors or patterns which can give rise to a pure strain species with the morphological appearance of a hybrid. This is especially problematic for isolated populations since variations in alleles due to chance mutation are more likely to go to fixation. Using morphology to determine a hybrid is further complicated when the hybrid backcrosses with an original parental species (as is common with naturally occurring hybrid populations). This can make the morphological differentiation of hybrids from parental species nearly impossible (Mallet 2005).

Due to the difficulties of identifying species based on taxonomy, biologists recently have turned to genetic techniques to determine the identity of hybrid populations. Genetic analysis allows a biologist to look beyond phenotypic characteristics that are under the pressure of natural selection (and thus cause polymorphisms within the species that can be confused for signs of a hybrid population). Some genetic techniques, such as assays of microsatellite DNA, use non-coding DNA that mutates at a steady background rate. This allows scientists to quantify degrees of relatedness among individuals or populations without concern about polymorphisms that are affected by natural selection. This also gives scientists the ability to infer the ancestral history of an individual or population since their DNA was passed down to them from previous generations (Beebe and Rowe 2004). One example of a commonly used genetic marker for such analyses is microsatellite DNA loci. Microsatellites are genetic loci that consist of variable number tandem repeats that range in size from 2-6 base pairs (Freeman and Herron 1998). Since microsatellites

are stretches of non-coding DNA that do not convey a phenotype, they are considered neutral markers (Freeland 2005). This makes microsatellites ideal for genetic analysis since they are not affected by natural selection or environmental changes.

An example of a possible natural hybridization event occurs in a population of massasauga rattlesnakes (*Sistrurus catenatus*) in Pershing State Park in north central Missouri. Massasaugas are a small species of rattlesnake (20-24 inches) that ranges from central New York to southeastern Arizona and the Gulf Coast of Texas. Three sub species of massasaugas exist: the eastern massasauga (*S. c. catenatus*), the western massasauga (*Sistrurus c. tergeminus*) and the desert massasauga (*S. c. edwardsii*) (Szymanski 1998). Massasaugas are highly localized in their habitat preferences: *S. c. catenatus* prefers environments in or adjacent to wetlands, *S. c. tergeminus* prefers drier prairie habitats (Stebbins 1966) while *S. c. edwardsii* prefers shortgrass prairies containing sand sage (*Artemisia filifolia*), buffalograss (*Buchloe dactyloides*) and blue grama (*Bouteloua gracilis*) (Mackessy *et al.* 2005). Populations of *S. c. catenatus* are highly fragmented due to urbanization and agriculture. Gibbs *et al.* (1997) found significant  $F_{ST}$  values among all sampled populations of *S. c. catenatus*, thus showing that these populations exhibit high levels of genetic differentiation. This is most likely due to the fragmentation and isolation of the *S. c. catenatus* populations combined with their small population sizes. *S. c. catenatus* is a candidate to be placed on the federal endangered species list (Moser 2006) and is afforded state-level endangered species protection across its entire range.

Evans and Gloyd (1948) argued that the Pershing population consisted of individuals which are hybrids between *S. c. catenatus* and *S. c. tergeminus*. They studied morphological traits such as variation in numbers of dorsal blotches on the body, number of ventrals, and “general coloration” for thirty five massasaugas. Evans and Gloyd also relied heavily on a subjective interpretation of the dorsal pattern on the head of massasaugas. They reported that the massasaugas from north-central Missouri had a head dorsal pattern that was a mix between that of *S. c. catenatus* and *S. c. tergeminus*. To complicate the study, Evans and Gloyd stated that there existed a possibility of erroneous locality records.

Evans and Gloyd’s 1948 classification of the Pershing population as a hybrid population did not last as this population is currently described as *S. c. catenatus*. This classification is based on a gradual change in morphology between *S. c. catenatus* and *S. c. tergeminus* described in Szymanski (1998). Due to this classification, the Pershing population has been afforded

endangered species status by the state of Missouri since 1974 (Johnson and Figg 1993). To add confusion to the Pershing population's species identity debate, Kubatko and Gibbs (unpublished data) suggested that this population's identity should be *S. c. tergeminus* based on ongoing phylogenetic analyses of DNA sequence data.

Due to this dispute over the population's identity, I decided to investigate the genetic identity of these massasauga rattlesnakes in Missouri by using microsatellite DNA. Microsatellites have been used in previous studies of snake population genetics by Bushar *et al.* (1998) and Clark *et al.* (2008) on timber rattlesnakes (*Crotalus horridus*) and Lougheed *et al.* (1999) on black ratsnakes (*Pantherophis alleghaniensis*). Gibbs *et al.* (1997) conducted the only detailed population genetic study on *S. c. catenatus*. They used six microsatellite loci to estimate genetic differentiation of several populations of *Sistrurus c. catenatus* in Ohio, New York, and Ontario. Advancements in genetic analysis since the Gibbs *et al.* (1997) study (such as automated microsatellite sequencing and microsatellite primer multiplexing) provide the possibility of collecting larger amounts of genetic data more efficiently. In addition, more powerful analysis methods (such as the Bayesian statistical analysis program STRUCTURE (Pritchard *et al.* 2000) and the phylogenetic suite of programs PHYLIP (Felsenstein 1993)) give evolutionary biologists powerful new tools to analyze microsatellite data.

Several studies specifically have used microsatellite data and the program STRUCTURE to analyze for the possibility of hybrid species (Trigo *et al.* 2008, Lancaster *et al.* 2006, Nielsen 2003). Here, I used microsatellite DNA to investigate the genetic identity of the Pershing population of massasauga rattlesnakes. Based on earlier unpublished work done by Kubatko and Gibbs as well as Pershing State Park's geographic proximity to *S. c. tergeminus* populations, I predicted that I would identify the Pershing population of massasauga rattlesnakes as *S. c. tergeminus*.

## **Methods:**

### *Sample locations and sampling techniques:*

I obtained DNA from 166 blood samples taken from the caudal vein of massasaugas captured from eight populations. I used known *S. c. tergeminus* blood samples collected from three populations in Kansas: Cheyenne Bottoms (n=20); Ellsworth county (n=11); Russell county (n=15); and one population in Missouri: Squaw Creek National Wildlife Refuge (n=34). I

used samples of *S. c. catenatus* from one population in Illinois: South Shore State Park (n=18); and two populations in Ohio: Killdeer Plains (n=25); and Clark County (n=21). The potential hybrid population from north-central Missouri is located in Pershing State Park (n=21) (Figure 1). All individuals were PIT-tagged to identify those caught multiple times. Blood samples were stored in lysis buffer and kept frozen at -20°C.

#### *Data collection techniques:*

I extracted DNA from blood samples using a phenol-chloroform protocol (Sambrook *et al.* 1989). DNA was stored in TE buffer after extraction and kept frozen at -20°C. I used microsatellite primers, loci, and PCR solutions and parameters developed by Anderson *et al.* (in prep) for *S. c. catenatus* to amplify the microsatellites. I found that seventeen of the nineteen primers described by Anderson *et al.* also amplified microsatellite loci in *S. c. tergeminus* and I used these loci for the project. I made two changes to the PCR parameters described by Anderson *et al.* in order to amplify *S. c. tergeminus* microsatellites: I ran Sca210 and Sca211 at an annealing temperature of 55°C and I doubled the volume of PCR solution for sequencer analysis of Sca217 and Sca218 to 4.0µL. I ran the PCRs on an ABI 3100 genetic analyzer and the program GeneMapper v3.7 (GeneMapper 2005) analyzed the data. I checked each microsatellite individually to ensure correct size calling of the alleles. I compiled this data into a spreadsheet and used the program CREATE (Coombs *et al.* 2008) to reformat the data into the correct format for each analytical program. Finally, I checked all microsatellite data for errors using MICROSATELLITE ANALYZER (MSA) to further verify the integrity of the compiled data set. (Dieringer and Schlötterer 2003).

#### *Data Analysis:*

I used two methods to evaluate the identity of the unknown central Missouri population. First, I used STRUCTURE (Pritchard *et al.* 2000), a cluster-based analysis program which uses a Bayesian approach to infer the percent of each individual's genome to a given cluster. I analyzed 166 individuals at K=2 populations with a burn in period of 100,000 repetitions followed by 1,000,000 Markov chain Monte Carlo (MCMC) repetitions from which the data was generated. I used the admixture model with correlated allele frequencies and calculated the 95%

probability intervals. I used the ln probability graph to insure the statistical analysis had run enough repetitions. Each run for a given K was repeated five times to ensure reliability of results.

Second, I preformed a distance-based phylogenetic analysis. I used MSA to calculate pairwise estimates of Nei's genetic distance both among individuals (n=166) and among the following three population groups: known *S. c. catenatus*, known *S. c. tergeminus*, and the unknown Pershing population. I used three programs from the PHYLIP suite (Felsenstein 1993) to create distance trees. FITCH created a distance tree via the Fitch-Margoliash method (Fitch and Margoliash 1967) for all 166 individuals and NEIGHBOR created 1000 neighbor-joining distance trees (Saitou and Nei 1987) from the bootstrapped population distance matrix. The bootstrapped trees were run through CONSENSE to find a majority consensus tree. I used FigTree (Rambaut 2007) to graphically display both trees. In addition to these analyses, I used MSA to create pairwise  $F_{ST}$  values to tell us the degree of genetic differentiation between each population.

## Results:

Both *S. c. catenatus* and *S. c. tergeminus* show evidence for genetic structure among populations within the species; however, the levels of genetic differentiation among the *S. c. catenatus* populations are greater than the level of genetic differentiation among populations of *S. c. tergeminus*. The average  $F_{ST}$  value between *S. c. tergeminus* populations and the Pershing population was 0.07 and for *S. c. catenatus* to the Pershing population was 0.23 (Table 2). However, when I *a priori* limit structure to only two distinct clusters, STRUCTURE clearly places the four known *S. c. tergeminus* populations (Cheyenne Bottoms, Ellsworth county, Russell county, and Squaw Creek National Wildlife Refuge) into cluster one and the three known *S. c. catenatus* populations (South Shore State Park, Killdeer, and Clark County) into cluster two (Figure 2). Individuals from the Pershing population cluster strongly with the *S. c. tergeminus* and have a mean  $q_1$  value (the average value of the proportion of an individual's genome assigned to cluster 1) of 0.976 (0.877-1.000 95% *P.I.*) (Table 1). A note of special interest is the single individual in the Pershing population that has a  $q_1$  value of 0.711 (0.442, 1.000 95%*P.I.*). The STRUCTURE results were consistent across five iterations with nearly identical average  $q$ -values and 95% probability intervals. The ln likelihood plot for all five trials

quickly went to fixation and remained fixed for the entire run (Figure 3). This shows a high degree of confidence that STRUCTURE was run for enough MCMC repetitions.

The distance tree of all 166 individuals distinctly separated the *S. c. tergeminus* individuals from the *S. c. catenatus* individuals. The tree groups individuals from Pershing, Missouri with *S. c. tergeminus*, even interspersing some of the known *S. c. tergeminus* individuals among the Pershing individuals (Figure 4). Based on bootstrap analyses, the neighbor joining majority consensus tree grouped the Pershing population with *S. c. tergeminus* 1000 out of 1000 times (Figure 5). As with the Structure analysis, there was one individual from the Pershing population that was placed between the Pershing population and the *S. c. catenatus* populations. This is the same individual which was given a  $q_1$  value of 0.711 by STRUCTURE.

## Discussion:

### *Interpretation of Results:*

Results from both cluster based analysis and phylogenetic distance-based analysis showed that the Pershing population of massasauga rattlesnakes grouped strongly with the known *S. c. tergeminus* populations. Cluster based analysis assigned the Pershing population a mean probability value of  $q_1=0.976$ . If one assumes a  $q$ -value of 0.9 as the threshold to distinguish pure strain species from hybrid individuals (Lancaster *et al.* 2006; Vähä & Primmer 2006; Oliveira *et al.* 2008; Trigo *et al.* 2008), then the Pershing population should be considered a pure strain species.

Phylogenetic distance-based analyses grouped the Pershing population with known *S. c. tergeminus* for all 1000 bootstrapped neighbor joining trees. This illustrates a high degree of confidence in the distance matrix and lends to the credibility of its results. The distance tree consisting of all 166 sampled individuals also grouped the Pershing individuals with known *S. c. tergeminus* individuals and even interspersed some individuals from known *S. c. tergeminus* populations among Pershing individuals. This distance-based phylogenetic analysis provides strong support that the Pershing population is much more closely related to *S. c. tergeminus* than *S. c. catenatus*.

Additionally, calculated pairwise  $F_{ST}$  values (Table 2) among Pershing and *S. c. tergeminus* populations are much lower ( $F_{ST}=0.07$ ) than between Pershing and *S. c. catenatus*



populations ( $F_{ST}=0.23$ ). These values add to the evidence that the Pershing population is genetically most similar to *S. c. tergeminus*. When taken as a whole, the body of evidence I have provided overwhelmingly shows that the species identity of the Pershing population of massasaugas in north-central Missouri is *Sistrurus catenatus tergeminus*.

While the population as a whole is composed of *S. c. tergeminus*, there is one potential hybrid individual in the Pershing population. STRUCTURE assigned this individual a  $q_1$  value of 0.711 and a 0.442, 1.000 95% *P.I.* A  $q$  value less than 0.9 and a broad 95% confidence interval are typical for hybrid individuals in a STRUCTURE analysis (Trigo et. al, Beaumont *et al.* 2001; Nielsen *et al.* 2003). However, it is possible that the STRUCTURE analysis reflected a portion of this individual's genome that is disproportionately related to *S. c. catenatus* compared to the individual's genome as a whole. This possibility could be ruled out if an independent genetic study, which uses a different portion of this individual's genome, yields results that give just as strong evidence for a hybrid individual as the STRUCTURE analysis provided. Without an independent genetic study, however, the genetic identity of this individual is left to speculation.

#### *Conservation implications:*

The results presented here contradict Evans and Gloyd's (1948) claim that the Pershing massasaugas were a hybrid population. My findings also contradict the current species designation by the USFWS that these snakes are *S. c. catenatus* (Szymanski 1998). To my knowledge, no published works have been done on the genetic identity of this population of massasaugas. Evans' and Gloyd's morphological work is the only published literature which tries to describe the species identity of this population of rattlesnakes. As discussed earlier, much of the literature written on possible hybrid reptile populations has been based on morphology, and this project is an example in which a genetic analysis reveals more than can be inferred from a morphological analysis. Therefore, this study also shows the value in using genetic analysis in species classification.

The natural habitat of *S. c. catenatus* has been decimated throughout its range. This has caused *S. c. catenatus* to be restricted to a handful of fractured and highly isolated populations. Gibbs *et al.* (1997) showed high levels of genetic differentiation exist among these populations. These high levels of genetic differentiation are an indicator of low amounts of gene flow among these populations. This makes *S. c. catenatus* more susceptible to localized extinction due to the

possibility of a reduction in genetic diversity after a selection event or due to genetic drift. If the population is able to rebound after such an event, the genetic diversity will remain low due to the non-availability of new alleles in the population (Freeland 2005). Due to these concerns, *S. c. catenatus* is currently listed as endangered at the state-level throughout its range and is listed as a candidate specie for endangered species status by the US Fish and Wildlife Service (Moser 2006). The habitat of *S. c. tergeminus* has not been degraded to the same extent as *S. c. catenatus* and this has resulted in greater numbers of *S. c. tergeminus*. Therefore, *S. c. tergeminus* is afforded no special protection at the state or federal level. As a result of reclassifying the Pershing population as *S. c. tergeminus*, these snakes no longer warrant the endangered status given to *S. c. catenatus*, and thus, one of the criteria for special protection of this population has been removed. Despite its new species identity, the Pershing population probably will remain under special protection by the state of Missouri due to the rarity of massasaugas in the state. I found that pairwise  $F_{ST}$  values among Pershing and Kansas *S. c. tergeminus* populations were significantly greater than pairwise  $F_{ST}$  values among the Kansas populations. I also found that when I ran STRUCTURE for values of  $K > 2$ , Pershing was identified as a separate population (unpublished data not included in this study). These two observations confirm that the Pershing population is significantly differentiated from the Kansas populations due to its isolation. Since the Pershing massasaugas are an isolated population with no possibility of gene flow with other *S. c. tergeminus* populations, it remains at an increased risk for local extinction due to the reasons discussed above and still warrants local protection. However, while state-level protection probably will remain unchanged, the new classification of the Pershing population as *S. c. tergeminus* would make it ineligible to benefit from any federal status changes to *S. c. catenatus*.

#### *The role of the Mississippi River as a barrier to gene flow:*

The results of this research also suggest important evolutionary implications about the role of geographical barriers in genetic differentiation. By classifying the Pershing population as *S. c. tergeminus*, the range of *S. c. catenatus* is confined to east of the Mississippi River. Soltis *et al.* (2006) compiled a review detailing literature which looked into the possibility of phylogeographical barriers in unglaciated eastern North America. This review cited multiple sources of reptile and amphibian genetic discontinuity between eastern and western clades separated by geographical barriers such as the Mississippi River (Leache & Reeder 2002,

Burbrink 2002, Burbink *et al.* 2000, Moriarty & Cannatella 2004, Austin *et al.* 2004, Hoffman & Blouin 2004, Lawson 1987). My classification of the Pershing massasaugas as *S. c. tergeminus* adds evidence to the hypothesis that the Mississippi river can act as a phylogeographic barrier since the river now effectively creates a border which separates the two species. Soltis *et al.* (2006) only listed three studies which investigated phylogeographical barriers in snakes: Burbrink 2002 (*Elaphe guttata*), Burbink *et al.* 2000 (*Elaphe obsoleta*), and Lawson 1987 (*Nerodia rhombifera* and *N. taxispilota*, which did not use genetic techniques). None of these studies were on rattlesnakes; therefore, this study also helps to fill a literature gap which contains only the above few cases of snakes being subject to phylogeographic barriers.

#### *Limitations of the study:*

Many unanswered questions remain about the evolutionary and ecological dynamics of these *Sistrurus* populations. One question that needs to be addressed is the difference in the level of population structure among populations of *S. c. tergeminus* versus those of *S. c. catenatus*. Gibbs *et al.* (1997) showed high pairwise  $F_{ST}$  values among multiple *S. c. catenatus* populations (some less than two kilometers apart). Pairwise  $F_{ST}$  values and phylogenetic distance-based analyses from my project confirm a high degree of structure among populations of *S. c. catenatus*. However, my  $F_{ST}$  and phylogenetic analyses of *S. c. tergeminus* showed much less differentiation than has been observed in *S. c. catenatus* populations. A variety of possible reasons exist for this difference including the degree of continuous habitat for *S. c. tergeminus* versus the more fractured habitats of *S. c. catenatus*. This hypothesis is complicated by the relatively low  $F_{ST}$  values among the Pershing population, which is physically isolated, and the other *Sistrurus c. tergeminus* populations versus the much higher  $F_{ST}$  values among *S. c. catenatus* populations with similar degrees of isolation. Therefore, a study that takes variables such as distance between populations, size of suitable habitat, effective population sizes, continuity of habitat between populations, and differences in habitat preferences between *S. c. tergeminus* and *S. c. catenatus* could help shed light on this issue.

In conclusion, the clustering analysis shows no evidence for hybridization and clusters the Pershing population strongly with the *Sistrurus catenatus tergeminus* populations. The phylogenetic and  $F_{ST}$  evidence show that the Pershing population is genetically most similar to *S. c. tergeminus*. This data demonstrates very strong evidence for classifying the Pershing

population of massasauga rattlesnakes as *S. c. tergeminus*. This population represents an example of using DNA-based genetic analyses to determine species identity in reptiles. The Pershing population was classified as a hybrid population based on morphology by Evans and Gloyd (1948) only to be refuted later by a hypothesis of a gradual variation in morphological characteristics between *S. c. catenatus* and *S. c. tergeminus* with the conclusion that the Pershing population belonged to *S. c. catenatus* (Szymanski 1998). By using microsatellite DNA that is not influenced by phenotypic polymorphisms and subjective criteria such as “general coloration” (Evans and Gloyd 1948), I convincingly have identified the Pershing population. As genetic techniques continue to be used in more studies on potential hybridization, scientists will surely find that the original ideas about many species’ identity and the relationships among these species will continue to be challenged and revised.

### **Acknowledgements:**

I would like to thank Dr. H Lisle Gibbs for taking me on as an undergraduate and guiding me in my research endeavors, Jimmy Chiucchi for the countless hours that he took out of his own research time to train, counsel and teach me, Jose Diaz for his support in the lab and Tony Fries and Christine Anderson for their help throughout my research. The Ohio State University College of Arts and Sciences provided financial support for this project.

### **Literature cited:**

- Anderson E (1948) Hybridization of the habitat. *Evolution*, **2**, 1–9.
- Anderson E (1949) *Introgressive Hybridization*. Chapman & Hall, London.
- Arnold M (1996) *Natural Hybridization and Introgression*. Princeton University Press, Princeton.
- Arnold ML. 1997. *Natural Hybridization and Evolution*. New York: Oxford Univ. Press. pp. 215
- Austin JD, Loughheed SC, Boag PT (2004) Discordant temporal and geographic patterns in maternal lineages of eastern north American frogs, *Rana catesbeiana* (Ranidae) and *Pseudacris crucifer* (Hylidae). *Molecular Phylogenetics and Evolution*, **32**, 799–816.
- Barton NH (2001) The role of hybridization in evolution. *Molecular Ecology*, **10**, 551–568.

- Beaumont M, Barrat EM, Gottelli D *et al.* (2001) Genetic diversity and introgression in the Scottish wildcat. *Molecular Ecology*, **10**, 319–336.
- Beebe, Trevor J.C. and Graham Rowe. *An Introduction to Molecular Ecology*. New York: Oxford University Press Inc., 2004
- Bette L. Willis, Madeleine J.H. van Oppen, David J. Miller, Steve V. Vollmer, and David J. Ayre (2006). The Role of Hybridization in the Evolution of Reef Corals. *Annual Review of Ecology, Evolution, and Systematics*, 37: 489-517.
- Burbrink FT (2002) Phylogeographic analysis of the cornsnake (*Elaphe guttata*) complex as inferred from maximum likelihood and Bayesian analyses. *Molecular Phylogenetics and Evolution*, **25**, 465–476.
- Burbrink FT, Lawson R, Slowinski JB (2000) Mitochondrial DNA phylogeography of the polytypic North American rat snake (*Elaphe obsoleta*): a critique of the subspecies concept. *Evolution*, **54**, 2107–2118. Burr BM, Page LM
- Bushar LM, Reinert HK, Gelbert L (1998) Genetic variation and gene flow within and between local populations of the timber rattlesnake, *Crotalus horridus*. *Copeia*, **1998**, 411-422
- Campbell, Jonathan A., Brodie, Edmund D. Jr., Barker, David G., and Price, Andrew H. (1989). An apparent natural hybrid rattlesnake and *Crotalus willardi* (*viperidae*) from the Peloncillo Mountains of southwestern New Mexico. *Herpetologica*, 45( 3), 1989, 344-349
- Carson, H.L. et al. (1975) *Drosophila* hybrids in nature: proof of gene exchange between sympatric species. *Science* 189, 806–807
- Clark RW, Brown WS, Stechert R, Zamudio KR (2008) Integrating individual behavior and landscape genetics: the population structure of timber rattlesnake hibernacula. *Molecular Ecology*, **17**, 719-730.
- Cook, Sherburne F. Jr. (1955). Rattlesnake hybrids: *Crotalus viridis* x *Crotalus scutulatus*. *Copeia* Vol. 2 (May 20, 1955), pp. 139-141.
- Crespin, L. et al. (1999) Asymmetrical introgression in a freshwater fish hybrid zone as revealed by morphological index of hybridization. *Biol. J. Linn. Soc.* 67, 57–72
- Della Torre, A. et al. (1997) Selective introgression of paracentric inversions between two sibling species of the *Anopheles gambiae* complex. *Genetics* 146, 239–244
- Dieringer, Daniel & Schlötterer, Christian (2003) Microsatellite analyser (MSA): a platform independent analysis tool for large microsatellite data sets. *Molecular Ecology Notes* 3 (1), 167-169
- Dobzhansky T. 1937. *Genetics and the Origin of Species*. New York: Columbia Univ.

Press. 364 pp.

Evans, Philip D. and Gloyd, Howard K. (1948) The Subspecies of the massasauga, *Sistrurus catenatus*, in Missouri. *Bulletin of the Chicago Academy of Sciences* 8.9: 225-232.

Felsenstein, J. 1993. PHYLIP (Phylogeny Inference Package) version 3.5c. Department of Genetics, University of Washington, Seattle.

Freeland, Joanna R. Molecular Ecology. Sussex, England: John Wiley & Sons, Ltd, 2005. 51-53, 114-121.

Freeman, Scott and Jon C. Herron. Evolutionary Analysis. New Jersey: Prentice-Hall Inc., 1998.

Fitch, W. M., and E. Margoliash. 1967. Construction of phylogenetic trees. *Science* 155: 279-284.

Gibbs, H. L., K. A. Prior, P. J. Weatherhead, and G. Johnson. 1997. Genetic structure of populations of the threatened eastern massasauga rattlesnake, *Sistrurus c. catenatus*: evidence from microsatellite DNA markers. *Molecular Ecology* 6:1123-1132.

*GeneMapper Version 3.7*. (2005) [Computer software]. Carlsbad, California: Applied Biosystems

Grant V (1971) *Plant Speciation*. Columbia University Press, New York.

Hoffman EA, Blouin MS (2004) Evolutionary history of the northern leopard frog: reconstruction of phylogeny, phylogeography, and historical changes in population demography from mitochondrial DNA. *Evolution*, **58**, 145–159.

J. A. Coombs, B. H. Letcher, K. H. Nislow. (2008) Create: a software to create input files from diploid genotypic data for 52 genetic software programs. *Molecular Ecology Resources* 8 (3), 578-580.

Johnson, T.R. and D.E. Figg. 1993. Massasauga rattlesnake conservation in Missouri. Pages 78-79 in B. Johnson and V. Menzies (eds.), *Proceedings of the International symposium and workshop on the conservation of the eastern massasauga rattlesnake Sistrurus catenatus catenatus*. Metro Toronto Zoo, West Hill, Ontario. 141 pp.2

Lancaster ML, Gemmell NJ, Negro S *et al.* (2006) Ménage à trois on Macquarie Island: hybridization among three species of fur seal (*Arctocephalus* spp.) following historical population extinction. *Molecular Ecology*, **15**, 3681–3692.

Lawson R (1987) Molecular studies of thamnophiine snakes. I. The phylogeny of the genus *Nerodia*. *Journal of Herpetology*, **21**, 140–157.

- Leache AD, Reeder TW (2002) Molecular systematics of the eastern fence lizard (*Sceloporus undulatus*): a comparison of parsimony, likelihood, and Bayesian approaches. *Systematic Biology*, **51**, 44–68.
- Lehman N, Eisenhawer A, Hansen K *et al.* (1991) Introgression of coyote mitochondrial DNA into sympatric North American gray wolf populations. *Evolution*, **45**, 104–119.
- Lougheed SC, Gibbs HL, Prior KA, Weatherhead PJ (1999) Hierarchical patterns of genetic population structure in black rat snakes (*Elaphe obsoleta obsoleta*) as revealed by microsatellite DNA analysis. *Evolution*, **53**, 1995–2001.
- Mackessy, S.P. (2005, December 12). Desert Massasauga Rattlesnake (*Sistrurus catenatus edwardsii*): a technical conservation assessment. [Online]. USDA Forest Service, Rocky Mountain Region. Available: <http://www.fs.fed.us/r2/projects/scp/assessments/massasauga.pdf> [21 May 2009].
- Mallet J. 2005. Hybridization as an invasion of the genome. *Trends Ecol. Evol.* 20:229–37
- Mank JE, Carlson JE, Brittingham MC (2004) A century of hybridization: decreasing genetic distance between American black ducks and mallards. *Conservation Genetics*, **5**, 395–403.
- Mayr E. 1963. *Animal Species and Evolution*. Cambridge: Harvard Univ. Press. 797 pp.
- Moriarty EC, Cannatella DC (2004) Phylogenetic relationships of the North American chorus frogs (*Pseudacris*: Hylidae). *Molecular Phylogenetics and Evolution*, **30**, 409–420.
- Moser, Melissa. “Eastern Massasauga Rattlesnake (*Sistrurus s. catenatus*) Candidate Conservation Agreement with Assurances (CCAA) for Rome State Nature Preserve, Ashtabula County” 25 August 2006. U.S. Fish and Wildlife Services. 22 March 2008 <<http://www.fws.gov/Midwest/endange>
- Nielsen EE, Hansen MM, Ruzzante DE, Meldrup D, Gronkjaer P (2003) Evidence of a hybrid-zone in Atlantic cod (*Gadus morhua*) in the Baltic and the Danish Belt Sea revealed by individual admixture analysis. *Molecular Ecology*, **12**, 1497–1508.
- Nolte AW, Freyhof J, Tautz D (2006) When invaders meet locally adapted types: rapid moulding of hybrid zones between sculpins (*Cottus*, Pisces) in the Rhine system. *Molecular Ecology*, **15**, 1983– 1993.
- Noor, M.A.F. *et al.* (2000) Gene flow between *Drosophila pseudoobscura* and *D. persimilis*. *Evolution* 54, 2174–2175
- Oliveira R, Godinho R, Randi E *et al.* (2008) Molecular analysis of hybridisation between wild and domestic cats (*Felis silvestris*) in Portugal: implications for conservation. *Conservation Genetics*, **9**, 1–11.

- Post, R.J. (1984) Natural interspecific hybridisation of *Simulium sanctipauli* s.l. with *Simulium squamosum* and *Simulium yahense* (Diptera: Simuliidae). *Tropenmed. Parasitol.* 35, 58–60
- Pritchard, Jonathan K., Stephens, Matthew and Donnelly, Peter. (2000) Inference of Population Structure Using Multilocus Genotype Data *Genetics* 155: 945–959
- Rambaut, Andrew (2007). FigTree (Version 1.2.2) [computer software]. Retrieved 8 May 2009. Available from <http://tree.bio.ed.ac.uk/software/figtree/>
- Rieseberg LH (1997) Hybrid origins of plant species. *Annual Review of Ecology and Systematics*, **28**, 359–389.
- Rohwer, S. et al. (2001) Plumage and mtDNA haplotype variation across a moving hybrid zone. *Evolution* 55, 405–422
- Saetre, G.P. et al. (2001) Speciation, introgressive hybridization and nonlinear rate of molecular evolution in flycatchers. *Molecular Ecology* 10, 737–749
- Saitou, N., and M. Nei 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* 4:406–425
- Schwartz MK, Pilgrim KL, McKelvey KS *et al.* (2004) Hybridization between Canada lynx and bobcats: genetic results and management implications. *Conservation Genetics*, **5**, 349–355.
- Seminoff, Jeffrey A., Karl, Stephen A., Schwartz, Tonia and Resendiz, Antonio (2003). Hybridization of the green turtle (*Chelonia mydas*) and hawksbill turtle (*Eretmochelys imbricata*) in the Pacific Ocean: indication of an absence of gender bias in the directionality of crosses. *Bulletin of Marine Science*, 73(3): 643–652
- Soltis, Douglas E., Morris, Ashley B., McLachlan, Jason S., Manos, Paul S. and Soltis, Pamela S. (2006) Comparative phylogeography of unglaciated eastern North America. *Molecular Ecology* **15**, 4261–4293.
- Stebbins GL (1959) The role of hybridisation in evolution. *Proceedings of the American Philosophical Society*, **103**, 231–251.
- Stebbins, R.C. 1966. A Field Guide to Western Reptiles and Amphibians. Houghton Mifflin Co., Boston, Massachusetts.
- Szymanski, Jennifer. (1998) Status Assessment for Eastern Massasauga (*Sistrurus c. catenatus*) 1998.
- Trigo T. C., Freitas T. R. O., Kunzler G., Cardoso L., Silva J. C. R., Johnson W. E., O'Brien S. J., Bonatto S. L. and Eizirik E. (2008) Inter-species hybridization among Neotropical cats



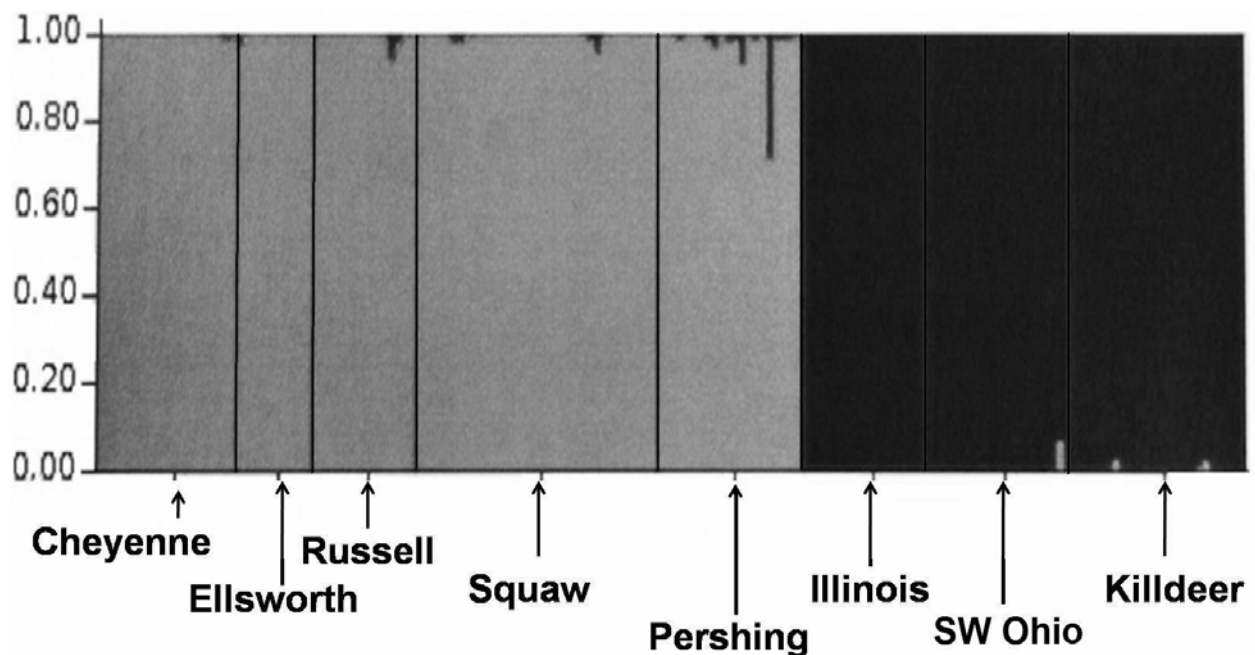
of the genus *Leopardus*, and evidence for an introgressive hybridzone between *L. geoffroyi* and *L. tigrinus* in southern Brazil. *Molecular Ecology* 17: 4317–4333

Vähä J, Primmer CR (2006) Efficiency of model-based Bayesian methods for detecting hybrid individuals under different hybridization scenarios and with different numbers of loci. *Molecular Ecology*, **15**, 63–72.

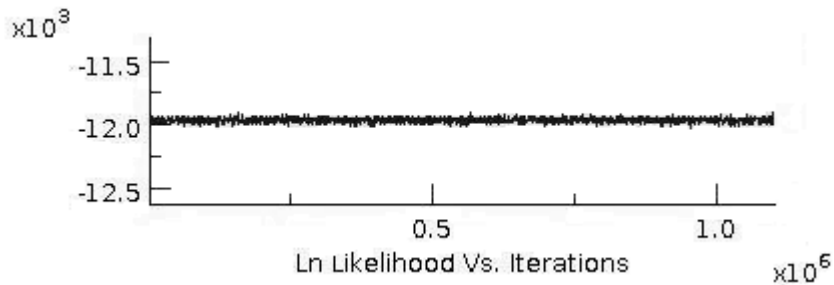
**Figure 1:** Map of the range and locals of the massasauga rattlesnakes (*Sistrurus catenatus*) sampled to determine the species identity of the Pershing massasaugas. The grey shading represents the range of *Sistrurus catenatus catenatus* and the black range is that of *Sistrurus catenatus tergeminus*. Stars are the locals from which massasaugas were collected. Black stars are populations of known *Sistrurus catenatus catenatus* and white stars are known *Sistrurus catenatus tergeminus* populations.



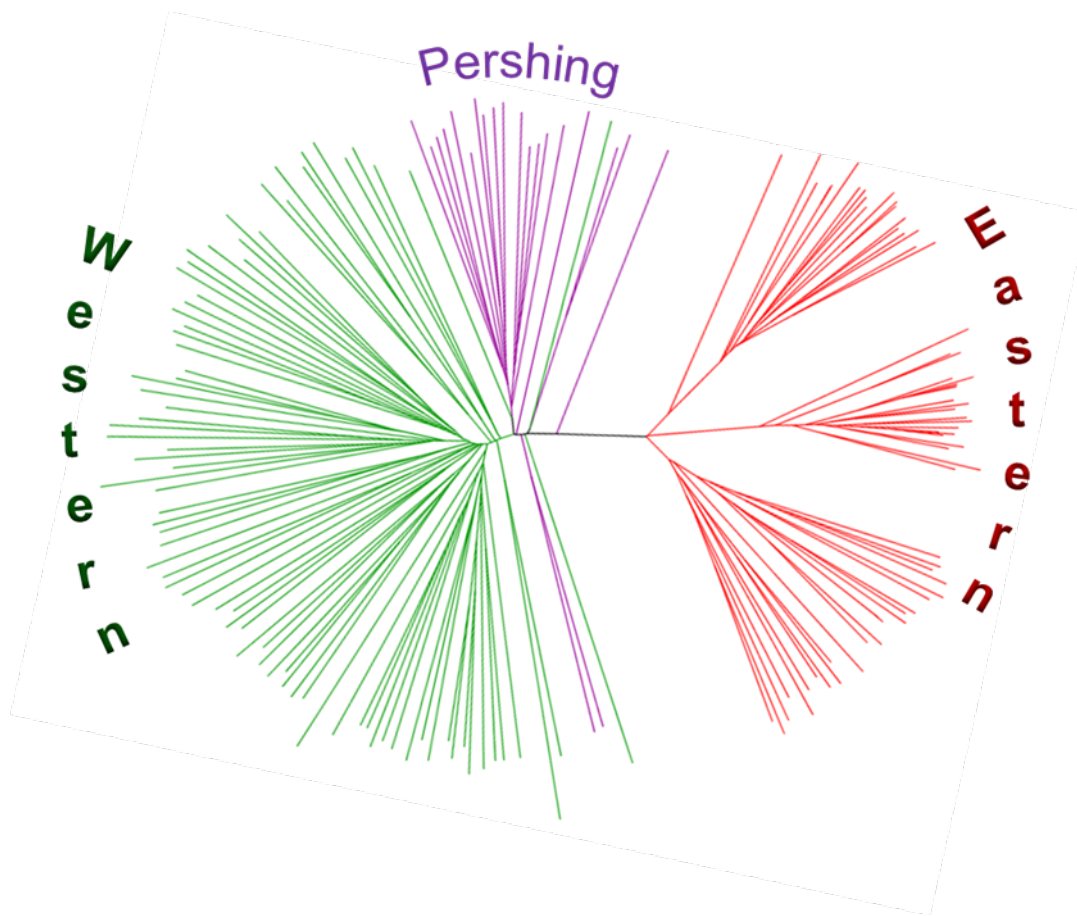
**Figure 2:** Graphical representation of STRUCTURE results for K=2 populations. The proportion of each massasauga's genome attributed to *Sistrurus catenatus catenatus* is black, and the proportion of each massasauga's genome attributed to *Sistrurus catenatus tergeminus* is grey. The individuals from the Pershing population strongly cluster with the *Sistrurus catenatus tergeminus* populations. This shows they are not hybrids since hybrids would be expected to have a greater proportion of black shading as well. This shows the Pershing population consists of *Sistrurus catenatus tergeminus*.



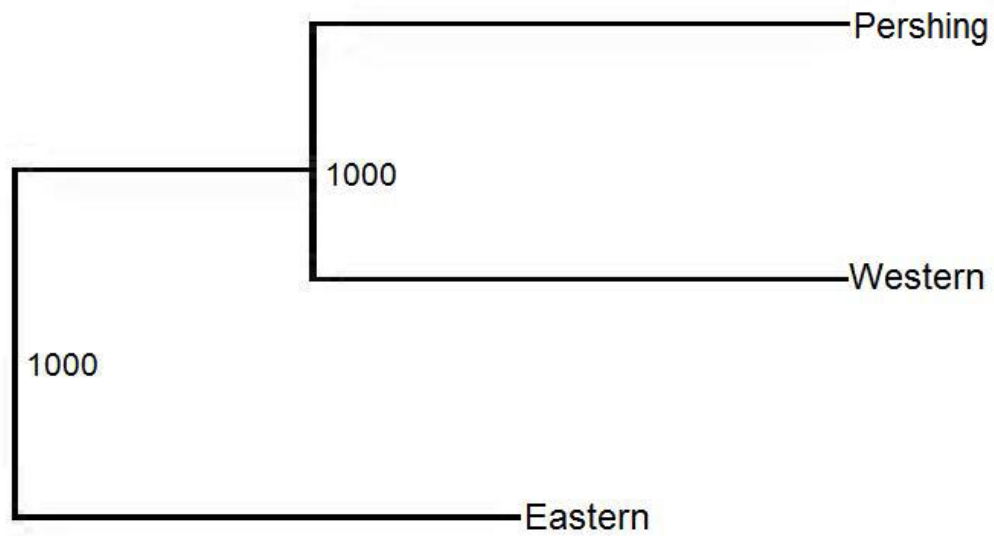
**Figure 3:** Graph of ln likelihood vs. number of MCMC repetitions for the STRUCTURE analysis performed to determine the species identity of the Pershing massasauga rattlesnakes (*Sistrurus catenatus*). Since no major variations exist in the height of the line, it can be assumed that enough burn in and MCMC repetitions were performed.



**Figure 4:** Neighbor-joining distance tree relating genetic distances of all n=166 massasaugas tested. *Sistrurus catenatus tergeminus* individuals are represented by green branches, *Sistrurus catenatus catenatus* individuals are represented by red branches, and the Pershing individuals are represented by purple branches. Massasaugas from Pershing clustered strongly with other western massasaugas. Note that several known western massasaugas are interspersed among the pershing population.



**Figure 5:** Majority consensus tree of 1000 bootstrapped neighbor-joining trees. Western massasauga (*Sistrurus catenatus tergeminus*) populations were Cheyenne, Russel, Ellsworth, and Squaw. Eastern massasauga (*Sistrurus catenatus catenatus*) populations were Illinois, Clark county, and Killdeer. The boot strap values show that the Pershing population grouped with western massasaugas in all 1000 distance trees.



**Table 1:** Inferred average clustering values (q-values) from STRUCTRE analysis for each population with the corresponding 95% probability interval. The four western massasauga (*Sistrurus catenatus tergeminus*) populations clustered strongly and the three eastern massasauga (*Sistrurus catenatus catenatus*) populations clustered strongly. The Pershing population clustered with the western massasauga populations very strongly which is evidence that the Pershing population is composed of *Sistrurus catenatus tergeminus*.

Population	q=1 (Western)	95% probability interval Western	q=2 (Eastern)	95% probability interval Eastern
Cheyenne	0.997	0.966-1.000	0.003	0.000-0.034
Ellsworth	0.996	0.964-1.000	0.004	0.000-0.036
Russell	0.993	0.951-1.000	0.007	0.000-0.049
Squaw	0.995	0.947-1.000	0.005	0.000-0.053
Pershing	0.976	0.877-1.000	0.024	0.000-0.123
Illinois	0.002	0.000-0.023	0.998	0.977-1.000
SW Ohio	0.005	0.000-0.028	0.995	0.972-1.000
Killdeer	0.005	0.000-0.056	0.995	0.943-1.000

**Table 2: A.)** The pairwise  $F_{ST}$  value between each population of massasauga rattlesnake is on the upper right of the diagonal and the corresponding P values with bonferoni correction are below and to the left of the diagonal.  $F_{ST}$  values are significant between each population except Russell and Cheyenne. This means that significant structure exists among all populations. **B.)** Average  $F_{ST}$  values among stated populations. The Pershing population was much less genetically differentiated from populations of *Sistrurus catenatus tergeminus* than from populations of *Sistrurus catenatus catenatus*. Also, less genetic structure exists among the *Sistrurus catenatus tergeminus* populations than the *Sistrurus catenatus catenatus* populations.

**A.)**

Population	Cheyenne	Ellsworth	Illinois	Killdeer	Pershing	Clark Co.	Russell	Squaw
Cheyenne	0.00	0.02	0.25	0.18	0.07	0.29	0.00	0.04
Ellsworth	0.0056	0.00	0.27	0.20	0.07	0.31	0.02	0.05
Illinois	0.0028	0.0028	0.00	0.17	0.23	0.27	0.24	0.24
Killdeer	0.0028	0.0028	0.0028	0.00	0.17	0.20	0.17	0.17
Pershing	0.0028	0.0028	0.0028	0.0028	0.00	0.27	0.07	0.07
Clark Co.	0.0028	0.0028	0.0028	0.0028	0.0028	0.00	0.28	0.28
Russell	not significant	0.0028	0.0028	0.0028	0.0028	0.0028	0.00	0.03
Squaw	0.0028	0.0028	0.0028	0.0028	0.0028	0.0028	0.0028	0.00

**B.)**

<i>S. c. tergeminus</i> populations excluding Pershing:	0.03
<i>S. c. tergeminus</i> populations including Pershing:	0.05
<i>S. c. catenatus</i> populations:	0.21
Pershing to <i>S. c. tergeminus</i> populations:	0.07
Pershing to <i>S. c. catenatus</i> populations:	0.23